

Localization of Neutralizing Epitopes and the Receptor-Binding Site within the Amino-Terminal 330 Amino Acids of the Murine Coronavirus Spike Protein

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To localize the epitopes recognized by monoclonal antibodies (MAbs) specific for the S1 subunit of the murine coronavirus JHMV spike protein, we have expressed S1 proteins with different deletions from the C terminus of S1. S1_{utt} is composed of the entire 769-amino-acid (aa) S1 protein; S1_{NM}, S1_N, S1_N(330), and S1_N(220) are deletion mutants with 594, 453, 330, and 220 aa from the N terminus of the S1 protein. The expressed S1 deletion mutant proteins were examined for reactivities to a panel of MAbs. All MAbs classified in groups A and B, those reactive to most mouse hepatitis virus (MHV) strains and those specific for isolate JHMV, respectively, recognized S1_N(330) and the larger S1 deletion mutants but failed to react with S1_N(220). MAbs in group C, specific for the larger S protein of JHMV, reacted only with the S1_{utt} protein without any deletion. These results indicated that the domain composed of the N-terminal 330 aa comprised the cluster of conformational epitopes recognized by MAbs in groups A and B. It was also shown that the epitopes of MAbs in group C were not restricted to the region missing in the smaller S protein. These results together with the fact that all MAbs in group B retained high neutralizing activity suggested the possibility that the N-terminal 330 aa are responsible for binding to the MHV-specific receptors. In investigate this possibility, we expressed the receptor protein and examined the binding of each S1 deletion mutant to the receptor. It was demonstrated that the S1_N(330) protein as well as other S1 deletion mutants larger than S1_N(330) bound to the receptor. These results indicated that a domain composed of 330 aa at the N terminus of the S1 protein is responsible for binding to the MHV-specific receptor.

Coronaviruses are enveloped, positive-stranded RNA viruses associated with various diseases of economic importance in both animals and humans (29, 31, 44). They cause a variety of acute and chronic diseases of the neurological, gastrointestinal, and respiratory systems (44). The murine coronaviruses are generally called mouse hepatitis virus (MHV), although there are many strains showing different organ tropism and disease characteristics (44). Some of them serve as animal models of virus-induced neurological diseases as well as models of various types of hepatitis (44).

MHV has a genome RNA of about 31 kb and encodes four major structural proteins: the 50- to 60-kDa nucleocapsid (N) protein, the 20- to 25-kDa integral membrane (M) glycoprotein, the 65-kDa hemagglutinin-esterase (HE) glycoprotein, and the 150- to 200-kDa spike (S) glycoprotein (29, 31). Several nonstructural proteins are encoded in the genome as well (29, 31).

As its name implies, the S protein constitutes the spike projecting from the virion surface (29, 42). The spike comprises two to three molecules of the S protein, each of which is a heterodimer consisting of two noncovalently bound S-protein subunits, S1 and S2 (31). These S1 and S2 subunits derive from the N-terminal and C-terminal halves of the S protein after cleavage of the precursor S protein by a host cell trypsin-like protease (35). It is believed that the S1 subunit forms the globular head of the spike and the S2 subunit forms its stalk portion (6). The S protein of MHV retains a variety of important biological activities. The S protein attaches the virus

to the cell surface by binding to MHV-specific receptors (15, 47). Although there are few reports concerning analysis of the receptor-binding site on the S protein, the topologies of the S-protein subunits suggest that the receptor-binding site is more likely to exist on S1 than on S2. The fusion of cultured cells infected with MHV is caused by the S protein (3, 38, 43). Uncleaved S protein has active fusion activity (32, 36), which suggests that the mechanism of MHV fusion formation differs from those of other fusogenic RNA viruses (46). The S protein is the major target of the neutralizing antibodies induced in mice after infection with MHV. It also elicits cytotoxic T cells (19). Furthermore, the S protein is suggested to be a major determinant of viral virulence in animals, although MHV virulence is influenced by numerous viral factors (4, 10, 12, 21). In spite of this variety of S-protein biological functions, little is known about the relationship between those biological functions and the structure of the S protein, the epitopes of monoclonal antibodies (MAbs) being an exception (5, 20, 25).

The MHV receptors are members of the carcinoembryonic antigen (CEA) family (7, 8, 47, 49). Expression of the receptor proteins in cells of nonmurine animal species, otherwise resistant to MHV infection, rendered those cells susceptible to the virus (7, 8, 49). For identification of the binding site of the S protein, which binds the host cell receptor, expression of the receptor protein should be extremely useful; a receptor protein expressed by DNA manipulation is much more abundant than the receptor protein existing on the susceptible cell lines.

In this study, we showed that the cluster of epitopes recognized by MAbs with neutralizing activity was localized within 330 amino acids (aa) from the N terminus of the S1 subunit. Furthermore, we demonstrated that this S1 domain bound to the cellular receptor. The S1 receptor binding experiments

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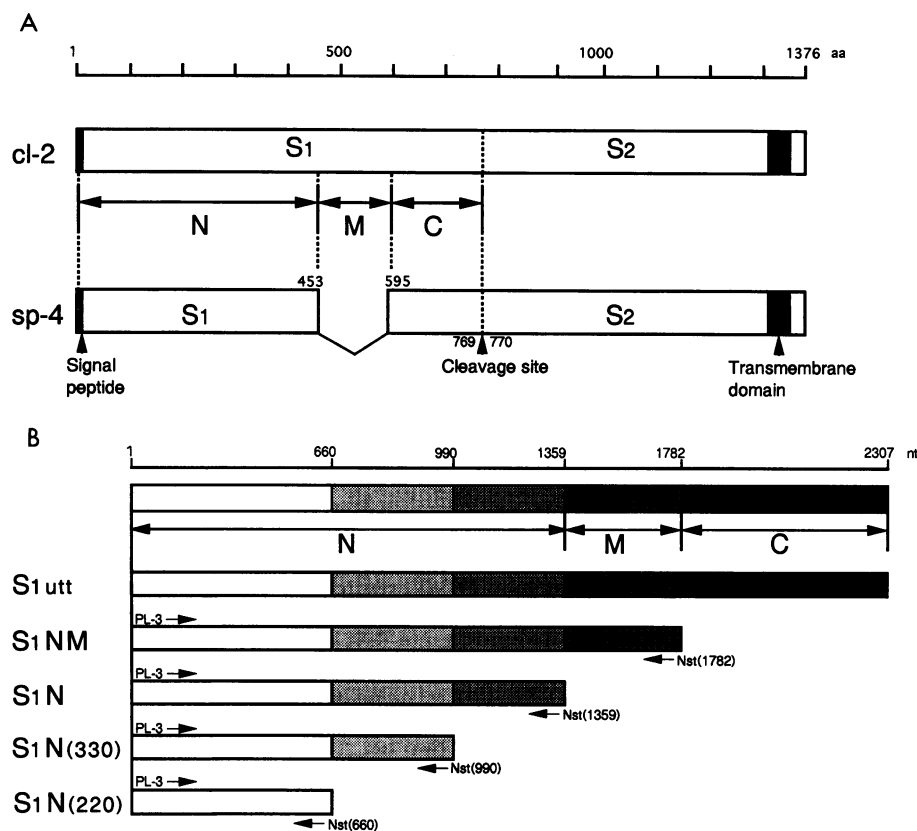


FIG. 1. (A) Structures of cl-2 and sp-4 S proteins. The cl-2 S protein is 1,376 aa long, and the S1 protein is 769 aa long. The sp-4 S1 protein has a 141-aa deletion designated M. The N- and C-terminal regions relative to the position of M were designated N and C, respectively. (B) Gene structures of the S1 deletion mutant proteins and positions of the oligonucleotides used for PCR. S1utt is composed of 2,307 nucleotides (nt) with additional 36 nucleotides at the 3' end (17). S1NM, S1N, S1N(330), and S1N(220) were composed of 1,782, 1,359, 990, and 660 nucleotides, respectively, from the first base of the initiation codon of the S protein. These genes were made by PCR with a pair of oligonucleotides as primers.

examined binding of deleted S1 proteins, which were progressively shortened from the C terminus, to receptor protein expressed by recombinant vaccinia virus (RVV).

MATERIALS AND METHODS

Viruses and cells. MHV strain JHMV cl-2 (39), plaque purified three times, was used for these experiments. The virus was propagated and assayed on DBT cells (14) as previously reported (40). Vaccinia virus (VV) strain WR, provided by A. Kojima, and its hemadsorption-negative mutant, provided by H. Shida, were used for producing RVV as well as for control virus infection. VV was propagated and assayed on RK13 cells (38) that were fed with Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo) containing 10% fetal calf serum (FCS; Gibco). DBT cells were grown in DMEM supplemented with 7% FCS and 10% tryptose phosphate broth (Difco).

Construction of plasmids. For expression of different C-terminal deletions of the S1 protein, we constructed various S1 genes that lacked successively longer segments from the 3' region. Construct S1utt covered the whole S1 gene encoding 769 aa with an additional 36 nucleotides and a termination codon in the 3' end, as previously reported (17). The S1NM, S1N, S1N(330), and S1N(220) genes encoding 594, 453, 330, and 220 aa from the N terminus of the S1 gene were constructed by PCR; cl-2 S cDNA (38) was the template, the forward primer was PL-3 (a positive-sense 30-mer oligonucle-

otide corresponding to the leader sequence of JHM, 5'-GCGTC-CGTACGTACCCTCTCTACTCTAAAA-3') (30), and the reverse primer was oligonucleotide S1N-Nst(1782) (5'-TTAT AAACAAGTGTGTCATGTGA-3'), S1N-Nst(1359) (5'-TTAAT TAAAACCATACCTCCT-3'), S1N-Nst(990) (5'-TTATTTA CAATCAGGTAGGTT-3'), or S1N-Nst(660) (5'-TTATTTA TCCGCATAGTACGC-3'), negative sense with nucleotides TTA complementary to the stop codon at the 5' end, as shown in Fig. 1B. We inserted the PCR products into pCR II from a TA cloning kit (Invitrogen, Valley Road, San Diego, Calif.) as instructed by the manufacturer and confirmed their sequences by the dideoxy-chain termination method (26). Each S1 gene was then cut from the vector with *EcoRI*, and the fragment was inserted into the *EcoRI* site of the VV transfer vector (VV-TV), pSF7.5EB1-B5-12 (pSF) (13), kindly provided by H. Shida. We selected the VV-TVs with S1 genes in the desired orientation and used them for transient expression of the S1 genes. The VV-TVs with S1utt, S1NM, S1N, S1N(330), and S1N(220) were designated pSFS1utt, pSFS1NM, pSFS1N, pSFS1N(330), and pSFS1N(220), respectively. The constructs of pSF containing the S1 gene with the transmembrane domain (S1tmd) or the S2 gene with the signal sequence (ssS2) were previously reported (17).

Transfection. For the transient expression of various S1 and S2 genes, we used DBT cells as previously described (17). DBT cells grown in 3-cm dishes (Falcon) to 70 to 80% confluence were infected with wild-type VV at a multiplicity of 1 to 2 PFU

per cell and incubated at 37°C for 1 h. Lipofectin (Bethesda Research Laboratories) and 2 to 3 µg of VV-TV DNA with various S genes were incubated as described previously (11, 17) and dropped into 1 ml of serum-free DMEM in which the infected cells were cultured. The cells were further cultured for 4 h, after which 1 ml of DMEM containing 20% FCS was added. At 20 to 24 h after VV infection, DBT cells were lysed, and the lysates were used for Western immunoblotting. For the receptor binding tests, we harvested culture fluids of VV-infected DBT cells, which were transfected with pSF carrying S1 deletion mutants, at 48 h after transfection.

Western blotting. Western blotting analysis was done mostly as reported previously (17, 36). DBT cells infected with VV and then transfected with VV-TVs were lysed at 20 to 24 h after VV infection with phosphate-buffered saline (PBS) containing 0.65% Nonidet P-40 and 2 mM phenylmethylsulfonyl fluoride (Sigma). As a control, DBT cells infected or mock infected with cl-2 virus were also lysed. The lysates were centrifuged at 15,000 rpm for 5 min at 4°C, and the supernatants were used as a source of various S1 deletion mutant proteins. An aliquot of supernatants was electrophoresed in a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel, and the proteins were electrically transferred onto Immobilon transfer membrane paper (Millipore). S. G. Siddell kindly provided the MAb that we used as a primary antibody for detection of the S1 deletion mutants; MAb 11F recognizes aa 33 to 40 from the N terminus of S1 (25). To detect the S2 protein, we used G10, also provided by S. G. Siddell (25). After incubation with the MAbs, the membrane papers were washed with PBS containing 0.5% Tween 20 and reacted with anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (Cappel Organon Teknica, Durham, N.C.) as a secondary antibody. The reaction was detected by enhanced chemiluminescence (Amersham).

Indirect immunofluorescence. VV- or cl-2-infected cells, mock-infected cells, and DBT cells expressing various S1 proteins, after infection with VV and transfection with pSF carrying various S1 genes, were fixed in acetone for 3 min at room temperature. Then they were incubated with the S1-specific MAbs produced in our laboratory (18) and 11F (25). After being washed with PBS, the cells were reacted with anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Cappel). Specific fluorescence in cells was detected by UV light microscopy.

Isolation of the MHV-specific receptor gene. The liver of a 6-week-old BALB/c mouse (Charles River Japan, Atsugi, Japan) was homogenized, and total RNA was extracted by the guanidium isothiocyanate method (2). The RNA was reverse transcribed into cDNA by using 200 U of reverse transcriptase (Bethesda Research Laboratories) with oligo(dT)₁₂₋₁₈ (Pharmacia) as a primer (48). The MHV receptor gene was then amplified (48) by a pair of primers that correspond to the published nucleotide sequences of mmCGM1 around the initiation and termination codons; the forward primer was MHVR-P(5') (5'-AGCAGAGACATGGAGCTGGC-3'), and the reverse primer was MHVR-N(3') (5'-TTGTCAGAAGGAGCCAGATC-3') (8). The amplified fragment with a length of about 900 bp (mL900) was purified in a 1% agarose gel and incorporated either in pCR II vector or downstream of the SRα promoter in the pCDL-SRα296 expression and cloning vector (41), kindly provided from Y. Takebe. The nucleotide sequence of mL900 was determined by the dideoxy-chain termination method.

Expression of the MHV receptor by RVV. The pCR II vector carrying mL900 was cut with *EcoRI*, and the 900-bp fragment was isolated by agarose electrophoresis. The fragment was

then inserted into the *EcoRI* site of the VV-TV, pSF, in the correct orientation. To isolate the RVV carrying an mL900-MHV receptor gene (RVV-MRe), we infected RK13 cells with wild-type VV and then transfected them with pSF carrying the receptor gene as previously reported (13, 38). After 40 to 48 h of incubation, the progeny viruses were harvested and the RVVs were selected for hemadsorption-negative phenotype and expression of receptor protein. Receptor protein expression was determined by indirect immunofluorescence with the rabbit anti-human CEA antibody (DAKO Japan, Kyoto, Japan) and FITC-conjugated anti-rabbit IgG (Cappel).

Binding assay of the S1 mutant proteins to the MHV receptor. By Western blotting, we examined the ability of the S1 mutant proteins to bind the receptor on prepared membranes. RK13 cells infected with RVV-MRe at a multiplicity of 1 PFU per cell were collected at 20 to 24 h postinfection and lysed. The lysates were electrophoresed on SDS-10% polyacrylamide gels and transferred onto transfer membrane paper as described above (17, 36). The membrane paper was pre-treated overnight with 25% Block Ace (Yukijirushi, Tokyo, Japan) at 4°C and incubated at room temperature for 1 h with test culture fluids; the culture fluids from DBT cells producing S1 deletion mutants resulted from infection of VV and transfection of pSF containing various S1 mutant genes. The membrane paper was then washed with PBS containing 0.5% Tween 20 and incubated with MAb 7 as well as other MAbs in groups A and B (18). Binding of MAb 7 was estimated by enhanced chemiluminescence (Amersham) with horseradish peroxidase-labeled anti-mouse IgG.

Inhibition test of receptor binding of the S1 by MAbs. The specificity of S1 binding to the MHV receptor was examined by testing the inhibition of binding by MAbs. Five hundred microliters of S1N(330) was mixed with 1 µl of neutralizing MAb 3, nonneutralizing MAb 2 [both of which are reactive with S1N(330)], MAb 5 (specific for the N protein), or PBS and incubated at room temperature for 50 to 60 min. The pre-treated S1N(330) was then incubated with membrane paper on which receptor protein had been prepared by Western blotting as described above. Binding of S1N(330) was then monitored by using MAb 7 as described above.

RESULTS

Expression of the S1 mutant proteins in DBT cells and their reactivities to a panel of MAbs. The MAbs specific for the S1 protein are largely classified into three groups: those that react with most MHV strains (group A), those that specifically react with JHMV (group B), and those that specifically bind JHMV variants with the larger S protein (group C). All of these MAbs recognize conformational epitopes (18). MAbs classified in group C do not react with the small S protein of sp-4 (37). JHM and sp-4 S1s have identically deleted S1 genes (27, 38), as shown in Fig. 1A. On the basis of this difference in the S protein between cl-2 and sp-4, we designated three regions of the S1 protein S1N, S1M, and S1C. The S1M region is missing in the small S protein of sp-4. The S1N and S1C regions correspond to the N- and C-terminal regions compared with the position of S1M (Fig. 1A). We constructed deleted S1 genes that expressed the S1NM (S1N plus M) and S1N proteins. Furthermore, we prepared two more S1 genes that expressed smaller S1 proteins, S1N(330) and S1N(220), which encoded 330 and 220 aa, respectively, from the S-protein N terminus. S1N(330) and S1N(220) were missing three and six hydrophilic regions located in the C terminus of S1N. These genes were obtained by PCR (Fig. 1B) and were inserted into the VV-TV, pSF. Each S1 deletion mutant was expressed in

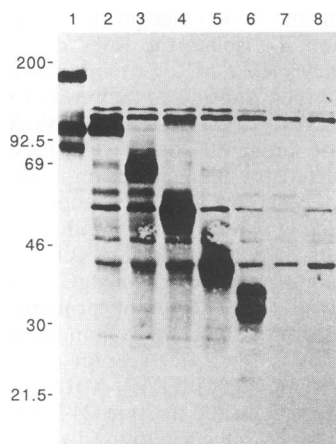


FIG. 2. Western blot analysis of the S1 deletion mutant proteins. DBT cells infected with VV were transfected with pSFS1utt (lane 2), pSFS1NM (lane 3), pSFS1N (lane 4), pSFS1N(330) (lane 5), or pSFS1N(220) (lane 6) or were mock transfected (lane 7), and cell lysates were prepared 24 h postinoculation. The lysates were also prepared from DBT cells infected with cl-2-infected (lane 1) or mock-infected (lane 8) cells. The lysates were electrophoresed in an SDS-polyacrylamide gel, and the proteins were transferred onto membrane paper. The paper was reacted with the S1-specific MAb 11F. The binding of 11F to the S1 mutants was detected by peroxidase-labeled anti-mouse IgG. Sizes are indicated in kilodaltons.

DBT cells by the VV transient expression system. For all constructs, indirect immunofluorescence revealed that 5 to 10% of DBT cells produced the S1 protein by 12 to 14 h posttransfection. To further analyze the S1 deletion mutants, DBT cell lysates were prepared 20 to 24 h after infection of wild-type VV and transfection with pSF plasmids containing S1 genes with different deletions. Expression of the S1 mutants was examined by Western blotting with MAb 11F, which recognizes a linear epitope in the N terminus of S1. As shown in Fig. 2 and Table 1, almost equivalent amounts of S1 deletion mutants were produced in cells transfected with various pSF plasmids, and the sizes of the expressed S1 proteins were in accord with the sizes deduced from their gene structures (Table 1). Cells transfected with pSFS1N(220) expressed two different proteins, of 35 and 31 kDa, which may have arisen from differential glycosylation. DBT cells expressing these species of S1 deletion mutants were examined for their reactivities to a panel of MAbs by indirect immunofluorescence. MAb 11F reacted to all mutant S1 proteins with similar intensities (Fig. 3). As shown in Table 2, all of the S1-specific

TABLE 1. Comparison of molecular weights of expressed S proteins

Recombinant plasmid	Core mol wt (10^3) ^a	No. of possible glycosylation sites	Deduced mol wt (10^3) ^b	Mol wt (10^3) detected by SDS-polyacrylamide gel electrophoresis
pSFS1utt	84.5	8		96
pSFS1NM	64.9	6	73.9	70
pSFS1N	49.7	5	57.2	54
pSFS1N(330)	35.7	4	41.7	41
pSFS1N(220)	23.5	4	29.5	35, 31

^a Obtained from computer analysis of each protein.

^b Calculated from core molecular weight and possible glycosylation sites.

MAbs reacted with S1utt in the same way as they did with the S protein produced in cl-2-infected DBT cells. All MAbs classified into groups A and B reacted with S1NM, S1N, and S1N(330). Group C MAbs reacted only with S1utt and not with any other S1 deletion mutants (Table 2). These results indicated that the epitopes recognized by MAbs in groups A and B are clustered in the S1 domain composed of 330 aa from the N terminus. In addition, all of the proteins except the smallest protein expressed by pSFS1N(220) form the same tertiary structure as does the entire S1 protein (18). Epitopes recognized by the MAbs in group C were not restricted to the S1M region that is missing in the small S protein.

Binding of S1 deletion mutants with the MHV-specific receptor. Most of the MAbs classified in group B showed very high neutralizing activity to cl-2 (18), which may suggest that these MAbs inhibit the binding of virus to the cellular receptor. If this is the case, the epitopes recognized by such MAbs or the regions adjacent to such MAb epitopes may play an important role in binding of the virus to the receptor. To investigate this possibility, we examined the binding of the S1 deletion mutants to the receptor. Several different protein species, including mmCGM1 (47) and mmCGM2 (49), function as MHV-specific receptors and arise by alternative splicing (7). Using a pair of primers based on the sequences of those proteins, we isolated the gene encoding the MHV receptor from mouse liver by reverse transcription PCR. The isolated MHV receptor was identical to BgpC or MHVR(2d) (7, 22). The expression of this protein in BHK-21 cells rendered the cells susceptible to MHV infection as previously described (7). We constructed an RVV with this MHV receptor gene, RVV-MRe, which produced large amounts of the receptor protein needed for S protein-receptor binding analysis. First, we examined the receptor protein by Western blotting. RK13 cells infected with the RVV-MRe were lysed at 20 to 24 h postinoculation, and the lysates were electrophoresed in SDS-polyacrylamide gels. The proteins were transferred onto membrane paper, and the MHV-specific receptor was detected with anti-human CEA antibodies by enhanced chemiluminescence. As shown in Fig. 4A, cells infected with the RVV-MRe produced a few different species of receptor proteins; a major band was at 41 kDa, and a few additional bands were around 46 kDa. These proteins were slightly larger than the mmCGM2 reported previously (49). We then examined the ability of these proteins to bind various S1 deletion mutants. MHV receptor-bound membranes were incubated with the lysates prepared from cells expressing S1 with the transmembrane domain (S1tmd) (17) or S2 with signal peptide (ssS2) (17). The membrane paper was also incubated with culture fluids from cells producing the S1 deletion mutant proteins via a VV transient expression system; these S1 mutants lacked the transmembrane domain and therefore was secreted mostly into the culture fluid. The binding of these S1 and S2 proteins was monitored with MAbs 7 and 10G, respectively. The S1 protein with a transmembrane domain bound to the 41- and 46-kDa receptor proteins, but binding of the S2 protein was indeterminate because of lack of reaction of MAb 10G even with the whole S protein bound to the receptor. Binding of the whole S protein could be easily detected by MAb 7 (data not shown). Figure 4B shows that S1N(330) as well as the larger S1 proteins bound to the receptor, while S1N(220) apparently did not. S1N(220) may have bound the receptor, because MAb 7, used for the detection of various S1 deletion mutants, failed to react with S1N(220). Also, MAb 11F, recognizing the N-terminal linear epitope of the S1 protein and reactive to all of the S1 deletion mutant proteins, failed to react with any of the S1 mutants when these mutant S1 proteins bound to the receptor. At

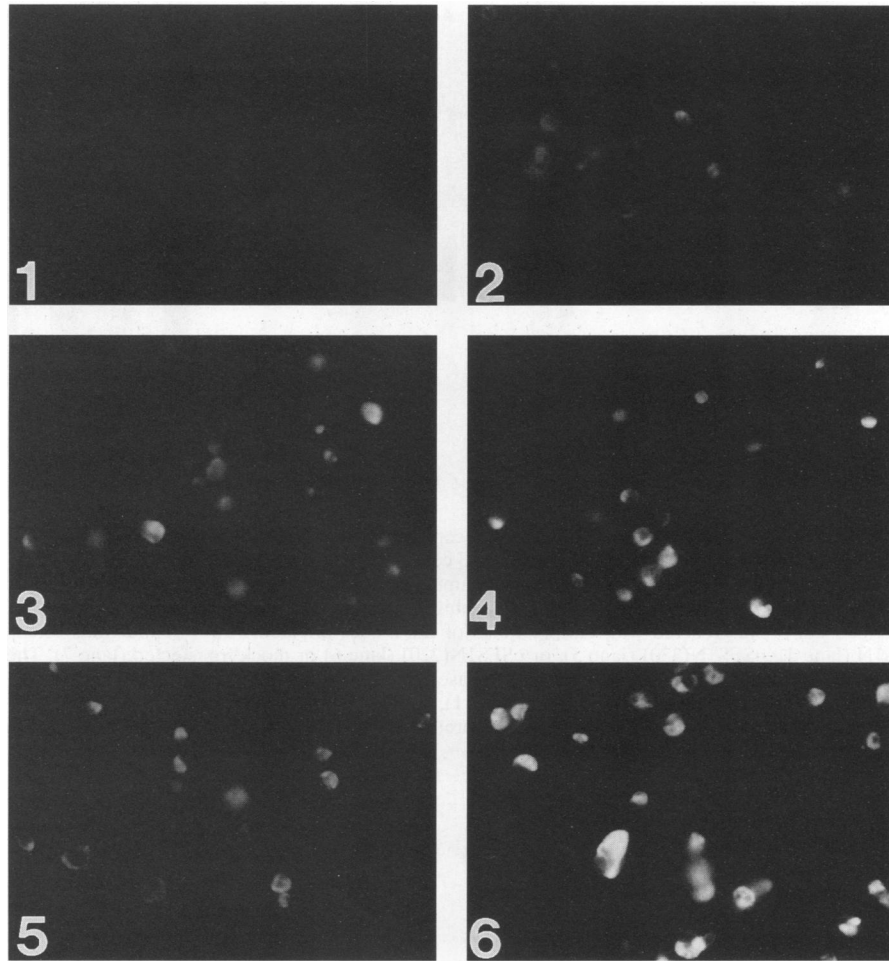


FIG. 3. Immunofluorescence analysis of the S1 deletion mutant proteins expressed in DBT cells. DBT cells infected with VV were mock transfected (lane 1) or transfected with pSFS1utt (lane 2), pSFS1NM (lane 3), pSFS1N (lane 4), pSFS1N(330) (lane 5), or pSFS1N(220) (lane 6). Cells were fixed 24 h postinoculation and reacted with 11F specific for S1 and then FITC-conjugated anti-mouse IgG.

present, we have no way to determine whether S1N(220) is capable of binding to the receptor. It was also evident from Fig. 4B that there were no striking differences in the amounts of the receptor-bound S1 mutant proteins except for S1N(220). These results clearly indicated that the region retained in the N-terminal 330 aa of the S1 protein is a major receptor-binding site in the S1 protein of MHV.

To examine the specificity of S1N(330) binding to the MHV receptor, we incubated S1N(330) with neutralizing MAb 3 or

nonneutralizing MAb 2, both of which are reactive with S1N(330). The treated S1N(330) was then monitored for binding to the receptor prepared on membrane paper. As shown in Fig. 4C, the binding of S1N(330) was inhibited by neutralizing MAb 3, while other nonneutralizing MAbs had no effect. These results clearly showed that the binding of S1N(330) to the MHV receptor was specific.

Reactivities of MAbs to the receptor-bound S1utt protein. We have examined the ability of the MAbs to bind the

TABLE 2. Reactivities of MAbs specific for the cl-2 S protein with the expressed S proteins^a

Recombinant plasmid	Reactivity													
	Group A MAb			Group B MAb					Group C MAb					
	2	7	19	3	6	13	71	93	8	12	47	63	78	85
pSFS1utt	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pSFS1NM	+	+	+	+	+	+	+	+	—	—	—	—	—	—
pSFS1N	+	+	+	+	+	+	+	+	—	—	—	—	—	—
pSFS1N(330)	+	+	+	+	+	+	+	+	—	—	—	—	—	—
pSFS1N(220)	—	—	—	—	—	—	—	—	—	—	—	—	—	—

^a The various S proteins were expressed in DBT cells infected with VV after transfection with pSF vectors containing various S genes. The reactivities were examined by indirect immunofluorescence.

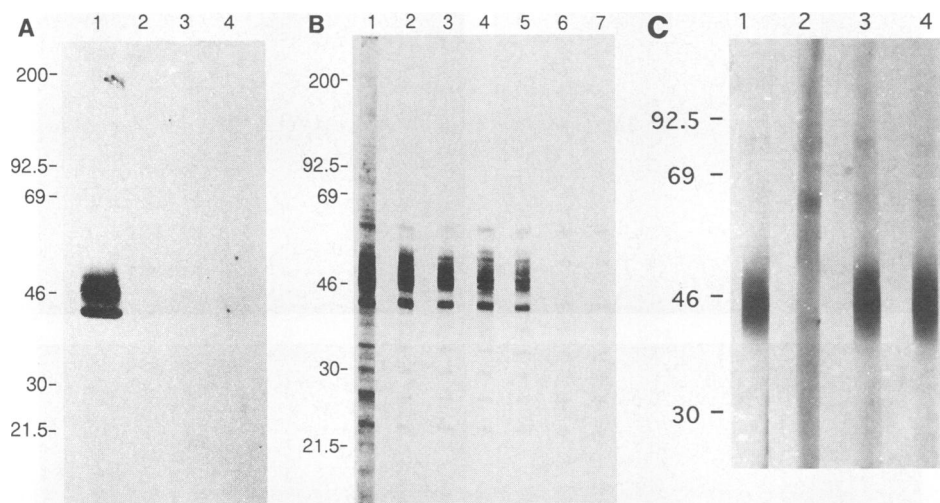


FIG. 4. Western blot analysis of the MHV receptor protein (A), receptor binding of the S1 deletion mutants (B), and inhibition of receptor binding of S1N(330) by MAbs (C). (A) The lysates prepared from RK13 cells infected with RVV-MRe (lane 1), VV (lane 2), or RVV-cl-2-S (lane 3) or mock infected (lane 4) were electrophoresed in an SDS-polyacrylamide gel, and the proteins were blotted onto membrane paper. The paper was reacted with anti-human CEA rabbit serum and then with anti-rabbit IgG conjugated with peroxidase. (B) The MHV receptor prepared on membrane paper as described above was reacted with culture fluids of DBT cells infected with VV and transfected with pSFS1utt (lane 2), pSFS1NM (lane 3), pSFS1N (lane 4), pSFS1N(330) (lane 5), or pSFS1N(220) (lane 6) or mock transfected (lane 7). The paper was then reacted with MAb 7 specific for the S1 protein and subsequently with peroxidase-labeled anti-mouse IgG. Lane 1 shows the position of MHV receptor protein detected as in panel A. (C) S1N(330) was mixed with PBS (lane 1), neutralizing MAb 3 (lane 2), nonneutralizing MAb 2 (lane 3), or anti-N MAb 5 (lane 4) and incubated at room temperature for 60 min. These pretreated S1N(330) proteins were monitored for receptor-binding capacity as described for panel B. Sizes are indicated in kilodaltons.

receptor-bound Slutt protein. The receptor protein prepared on membrane paper was reacted with Slutt as described above, and the binding of Slutt was monitored by using MAbs classified into groups A and B. All of the MAbs used showed almost same reactivity to the S1 protein upon mild-denatured Western blotting (17). As shown in Fig. 5, MAbs 2 and 7 in group A, both of which showed no virus neutralization activity, strongly reacted with the receptor-bound Slutt, while the binding of other MAbs was weak or not detected. Of the latter MAbs, all except MAb 19 belonged to group B and showed very high virus neutralization activity (1:100,000 to 1:500,000) with the exception of MAb 71 (1:2,000) (18). These results may indicate that binding of the neutralizing MAbs to the S1 protein was prevented by the receptor protein. Alternatively, the epitopes on the S1 protein recognized by the neutralizing MAbs may be involved in the receptor-binding activity of the S protein.

DISCUSSION

The S protein is involved in a variety of MHV biological functions: binding to the MHV-specific receptor, induction of fusion, elicitation of neutralizing antibodies and cytotoxic T cells, and pathogenesis of the virus (15, 31). However, very little is known about the relationship between such biological functions and the structure of the S protein except for the localization of neutralizing epitopes recognized by the MAbs (5, 20, 25) and fusion activity by molecularly engineered S protein (32, 36). The S protein has long been assumed to be involved in receptor binding. In this report, we showed that the clusters of antigenic epitopes exist within 330 aa from the N terminus of the S protein. Furthermore, an S1 deletion mutant protein composed of the N-terminal 330 aa of S1 bound to the MHV-specific receptor; this is the first evidence that the N-terminal region of the S protein plays an important role in

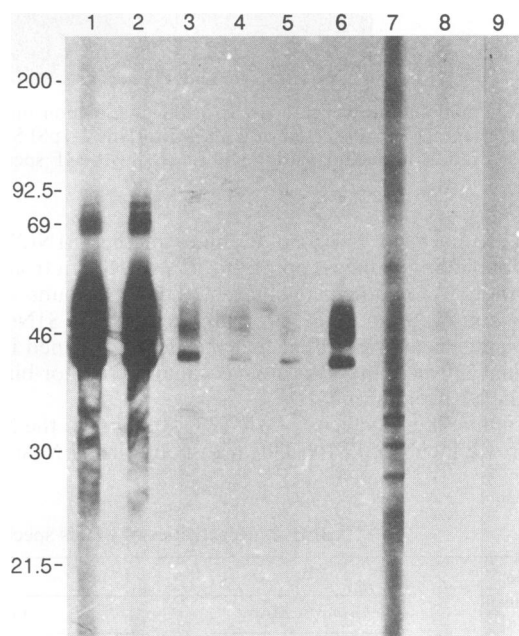


FIG. 5. Binding of MAbs to receptor-bound Slutt protein. Receptor protein prepared on membrane paper was reacted with the Slutt protein as described in the legend to Fig. 4. Receptor-bound Slutt was then reacted with MAbs classified in group A (MAbs 2 [lane 1], 7 [lane 2], and 19 [lane 3]) or group B (MAbs 3 [lane 4], 6 [lane 5], 13 [lane 6], 71 [lane 7], and 93 [lane 8]) or with culture fluid of DBT cells (lane 9). The binding of these MAbs to Slutt was examined by using peroxidase-labeled anti-mouse IgG.

receptor binding. This finding agrees with the idea that S1 comprises the bulbous portion of the characteristic club-shaped spike that is located in the outer extremity of the virion (6).

Various S1 deletion mutants larger than S1N(330) bound to the receptor proteins without striking differences in intensity of binding. This excludes the possibility that some region in the S1, other than the N-terminal 330 aa, is a major active site for receptor binding. The antigenic epitopes recognized by group B MAbs are unlikely to be centers of receptor-binding sites, because these antigenic epitopes are specific for JHM strains and the receptors are known to serve as targets for JHM and A59 (8, 49). The MAbs in group B probably recognize the epitopes existing in the neighboring region of the receptor-binding site on the S protein and inhibit virus binding to the receptor by steric hindrance. The receptor also served as a binding site for some other MHV strains besides JHM (unpublished data), suggesting that the receptor-binding active site is conserved within 330 aa from the S1 N terminus in most MHV strains. We are currently determining the smallest conserved amino acid sequence that functions as a receptor-binding site.

Work on different coronaviruses suggests the involvement of the S2 subunit in receptor-binding activity; MAbs specific for S2 of MHV have neutralizing activity (5, 45). However, the ability of S2 antibodies to neutralize virus may be the effect of steric hindrance, since the S protein is known to have a highly conformational structure (15, 31). Moreover, virus neutralization by antibodies does not always result from the prevention of virus binding to the receptor. When the S1 subunit of avian infectious bronchitis virus is removed, the virion is still able to bind susceptible cells, although it is not infectious (1). This finding demonstrated that the S2 subunit mediates attachment. However, this attachment did not trigger the replication of attached viruses and would not be the process that occurred with infectious virions retaining the S1 subunit. In the present study, we failed to demonstrate whether S2 bound to the receptor, because MAb 10G and others reactive to S2 in Western blotting were not effective for detection of the receptor-bound S proteins. At present, we have no data to rule out the possible receptor-binding capacity of the S2 protein.

The protein composed of the N-terminal 220 aa, S1N(220), did not react with any of the MAbs prepared in our laboratory; S1N(220) reacted with 11F, which recognizes the linear epitope composed of 8 aa from aa 33 to 40 of the S1 protein (25). It is not known whether the MAb failed to react with S1N(220) because all of the MAb's epitopes exist from aa 221 to 330 from the S1 N terminus or because S1N(220) could not form the conformational epitopes as it does in the entire S1 protein. We sequenced some S-gene mutant viruses that escaped from neutralization by our group B MAbs and determined that the MAbs recognized the region within 220 aa from the N terminus of the S1 protein (data not shown). This finding suggests that S1N(220) could not form the same secondary or tertiary structure as does the entire S protein; possibly the major receptor-binding site is located in the N-terminal 220 aa and functions in only a specific configuration. MAb 11F reacted with S1N(220) as well as other S1 mutant proteins by Western blot analysis (Fig. 2) but failed to react with the S1 mutant proteins bound to the receptor. This may indicate that the epitope recognized by 11F is either involved in the receptor binding or located in the region adjacent to the receptor-binding site of S1.

Since we could not test the binding capacity of S2 or S1N(220) by the method used in this study, we tried without success to detect it by preparing S2 or S1N(220) protein on

membrane paper by Western blotting and incubating the paper with the receptor protein prepared from cell lysates. By this method, even S1utt, which has been shown to be able to bind to the receptor protein by the method used in this study, did not bind to the receptor protein (data not shown). These data may indicate that the denatured S1 protein loses its binding capacity; i.e., the secondary or tertiary structure of the S1 protein is necessary for receptor binding. The conformational structure of the S1 protein has also been shown to serve as epitopes of the MAbs isolated in this laboratory (17). Thus, most of the biological functions of the S protein may be related to the conformational structure of the S protein.

In picornaviruses, a small canyon formed by the outer coat protein is proposed to be the precise location of receptor binding; this idea is the so-called canyon hypothesis (24). Members of the immunoglobulin superfamily serve as the receptors of these viruses (23, 34). The MHV receptors mmCGM1 and mmCGM2 are also members of the immunoglobulin superfamily (47, 49). There is another resemblance between picornavirus receptors and MHV receptors: their N-terminal domains, which are similar to the immunoglobulin variable region, are critical for interaction with viral proteins (9, 16, 28, 33). The mechanism of MHV virus-receptor binding may be similar to that of picornaviruses. Analysis of the receptor-binding site of the S protein will delineate the mechanism of virus-receptor interaction, which initiates virus infection.

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